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Increase in the Adhesion Molecule P-Selectin in Endothelium Overlying Atherosclerotic Plaques

Coexpression with Intercellular Adhesion Molecule-1

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P-selectin (GMP-140) is an adhesion molecule present within endothelial cells that is rapidly translocated to the cell membrane upon activation, where it mediates endothelial-leukocyte interactions. Immunohistochemical analysis of human atherosclerotic plaques has shown strong expression of P-selectin by the endothelium overlying active atherosclerotic plaques. P-selectin is not, however, detected in normal arterial endothelium or in endothelium overlying inactive fibrous plaques. Color image analysis was used to quantitate the degree of P-selectin expression in the endothelium and demonstrates a statistically significant increase in P-selectin expression by atherosclerotic endothelial cells. Double immunofluorescence shows that some of this P-selectin is expressed on the luminal surface of the endothelial cells. Previous work has demonstrated a significant up-regulation in the expression of the intercellular adhesion molecule-1 in atherosclerotic endothelium and a study on the expression of intercellular adhesion molecule-1 and P-selectin in atherosclerosis shows a highly positive correlation. These results suggest that the selective and cooperative expression of P-selectin and intercellular adhesion molecule-1 may be involved in the recruitment of monocytes into sites of atherosclerosis. (Am J Pathol 1994, 144:952–961)

In atherosclerosis, the adhesion of circulating monocytes to the endothelial lining is the earliest detectable event after cholesterol feeding in experimental animal models,[1] and the active stages of atherosclerosis are marked by the extensive infiltration of these blood-derived monocytes through the endothelium into the arterial intima.[2–4] It is now well established that endothelial adhesion molecules play an important part in the emigration of leukocytes from the blood into foci of inflammation,[5] and evidence is accumulating that similar processes are at work in atherosclerosis.

We have previously established that the intercellular adhesion molecule, ICAM-1, is extensively expressed on intimal cells in human atherosclerosis, including up-regulation in the plaque endothelium.[6] ICAM-1, is constitutively expressed on most types of endothelium, but in normal arteries there is relatively little expression. The adhesion molecule E-selectin has also been found to be increased in human atherosclerotic lesions.[7,8] In the rabbit, vascular cell adhesion molecule-1 is seen to be up-regulated in the endothelium overlying plaques,[9] but the evidence is conflicting in human atherosclerosis.[8,10]

This study investigates the expression of a further adhesion molecule, P-selectin, previously known as platelet activation dependent granule-external membrane protein (PADGEM) or granule membrane protein-140 (GMP-140), a member of the selectin family of cell surface adhesion molecules, which mediate the interaction of leukocytes with the endothelial lining of blood vessels. The primary structure of P-selectin has been deduced from complementary DNA cloning, and as with the other members of the selectin family of adhesion molecules, it is seen to be a protein with multiple domains, including a lectin domain, an epidermal growth factor domain, nine consensus repeats related to complement binding proteins, a transmembrane domain, and a short cytoplasmic region.[11] P-selectin is co-localized with von Willebrand factor (vWF) in the Weibel–Palade

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bodies of endothelial cells[12,13] and is rapidly mobilized from these granules to the cell surface upon activation by agonists such as thrombin, PMA, histamine, complement, and peroxides.[14,15] After surface expression on activated endothelium, P-selectin is rapidly reinternalized.[14]

The interaction of P-selectin with its ligand is calcium-dependent,[16] and several different ligands have been identified to bind P-selectin; the Lewis x antigen (Le-x),[17] sialyl-Le-x,[18] sulphated glycans,[19] and sulphatides.[20] The Le-x and sialyl-Le-x groups are oligosaccharides that are widely distributed on both glycoproteins and glycolipids of various cell types, including monocytes and neutrophils.

Many reports have suggested the involvement of P-selectin and ICAM-1 in the capture and migration of neutrophils into sites of inflammation.[21–26] Although the role of P-selectin in monocyte traffic has not been analyzed in detail, these cells carry the P-selectin ligand and may bind to the endothelium in a similar manner to neutrophils. As with neutrophil recruitment, ICAM-1 expression alone is unlikely to account for monocyte migration, as small vessels constitutively express ICAM-1 and this molecule seems to be incapable of supporting cell adhesion under flow conditions. P-selectin, however, has been demonstrated to support the rolling adhesion of neutrophils to endothelium, which is then reinforced by stronger ICAM-1-mediated bonds once the cells are stationary.[22]

In this account, we show that P-selectin expression is elevated in atherosclerotic endothelium and that it co-distributes with ICAM-1.

Materials and Methods

A series of arterial sections obtained from surgery (carotid artery obtained from reconstructive surgery $n = 11$) and postmortem (carotid and coronary arteries $n = 19$) were used in this study. Previous studies have shown good antigenic preservation in postmortem tissue up to 72 hours,[27] and no tissue over 48 hours postmortem was used in this study. The artery segments were snap-frozen in liquid nitrogen-cooled isopentane and serial 5-mm cryostat sections cut onto silane treated 4-spot slides. These sections were acetone fixed and stored at −70 C until use.

Two P-selectin (CD62) specific monoclonal antibodies were used in the analysis of the arterial sections. In addition, a mouse myeloma immunoglobulin, MOPC21, which lacks antigenic specificity, was used at comparable immunoglobulin concentrations to control for nonspecific binding. A platelet-specific antibody was also used to determine whether the positive staining seen in the arteries could be attributed to adherent platelets on the endothelium. The characteristics and sources of the antibodies used are listed in Table 1.

The avidin-biotin complex (ABC) immunoperoxidase technique was employed in this study. The sections were preincubated with 0.01% avidin solution followed by 0.01% biotin solution to block endogenous biotin. This was followed by 10% nonimmune rabbit serum, followed by the primary antibody for 90 minutes and the secondary biotinylated antibody. Endogenous peroxidase activity was then blocked by a 30′ incubation with glucose oxidase/glucose solution,[28] and finally the ABC was added and the reaction developed using diaminobenzidine as chromogen. The sections were counterstained with hematoxylin, dehydrated, and mounted.

All antibodies and conjugates were titrated on inflamed tonsil to determine the optimum concentrations for use. The concentration of mouse immunoglobulin in the LYP20 monoclonal antibody was determined by sandwich enzyme-linked immunosorbent assay, as described previously.[6]

Characterization of Lesions

The lesions of the series were characterized by the use of a panel of cell-specific markers (Table 1). Atherosclerotic plaques in the tissue were characterized on the basis of their morphology and the intimal macrophage and smooth muscle content. Endothelial integrity was confirmed by staining with vWF, a well-recognized endothelial marker, by the indirect immu-

Table 1. *Antibodies Used in the Study*

Specificity	Clone	Working Conc.	Source/Ref.
P-Selectin (CD62)	CBL-Thromb/6	10 μg/ml	Serotec
P-selectin (CD62)	LYP20	11 μg/ml	Ref. 29
von Willebrand factor	Polyclonal	1/2,000	Dako
GP1b	AN51	0.35 μg/ml	Dako
Mouse IgG1	MOPC21	10 μg/ml	Sigma
ICAM-1 (CD54)	15.2	0.12 μg/ml	Dr. N. Hogg, London
Smooth muscle α-actin	HHF35	0.7 μg/ml	Prof. A Gown, Seattle
Pan macrophage (CD68)	EBM11	3.5 μg/ml	Dako

noperoxidase method. The cell-specific staining and morphology allowed grouping of the lesions into subtypes[6]; as follows: fatty streak; fibro-fatty lesion; complex/advanced—these were essentially fibro-fatty but with disordered histology, neovascularization, calcification, or thrombosis; fibrous—consisting largely of smooth muscle and much dense connective tissue but with little or no macrophage involvement (these plaques may be an inactive end stage of atherosclerosis). Normal arteries and nonlesional areas of arterial intimas without eccentric thickening or significant macrophage infiltration served as controls.

Image Analysis

Staining of the endothelial region of the arterial sections was analyzed quantitatively by the use of hue-saturation-intensity color image analysis (Sight Systems, Hove, Sussex, UK; software by Foster Findlay Associates, Newcastle upon Tyne, UK), as previously described.[6] The hue-saturation-intensity system has the advantage over conventional red, green, and blue-based color image analysis in that it can be made relatively insensitive to light intensity, which may be liable to fluctuation.[30]

The arterial section for analysis was displayed on a color monitor as a digitalized image, using the 40× objective. Staining of the innermost intima in areas of intact endothelium (as demonstrated by positive vWF staining) was measured by setting the area for analysis as a strip 10 pixels in width, equivalent to 4.5 μ, the average thickness of the endothelial layer. Segments of intima approximately 160 μ in length were analyzed. The total area detected as positively stained was measured and expressed as a percentage of the area analyzed. Usually six measurements were made of each plaque, and the mean of these values used in the analysis.

Double Staining

Immunoperoxidase

The simultaneous detection of two cellular epitopes was achieved by double staining and was used to confirm the endothelial specificity of P-selectin expression. This method employed the

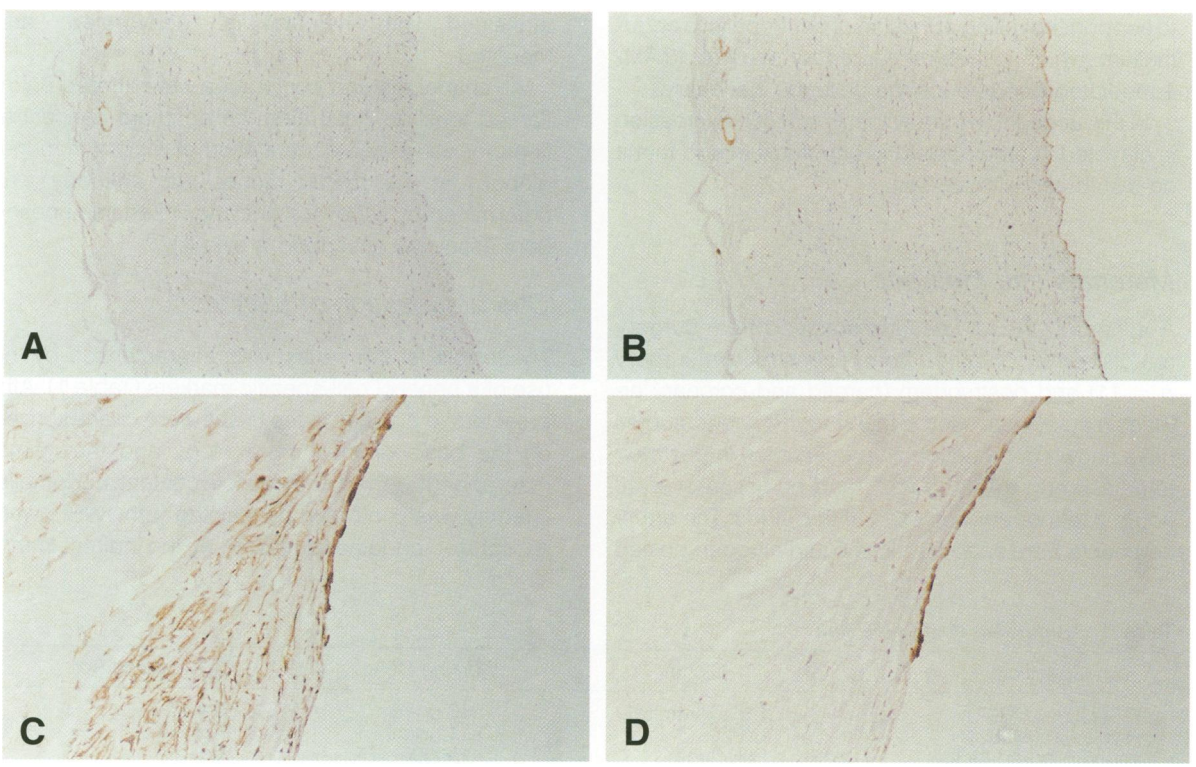

Figure 1. *Serial arterial sections stained by ABC immunoperoxidase showing a normal artery stained for P-selectin (LYP20,* **A**), *showing the absence of expression by the intimal endothelium but positive staining of the vasa vasorum and vWF* (**B**), *showing the integrity of the intimal endothelium. Original magnification 100×.* **C** *and* **D** *are serial sections of the shoulder of a fibro-fatty plaque, showing the correlation of endothelial expression of ICAM-1* (**C**) *and P-selectin* (**D**). *Strong endothelial staining can be seen at the edge of the plaque and an absence of staining over the fibrous cap. The endothelial integrity was demonstrated in a further serial section stained with vWF (not shown). Original magnification 200×.*

combination of rabbit polyclonal (vWF) and mouse monoclonal (LYP20) primary antibodies and the indirect and ABC immunohistochemical techniques respectively, with the reaction products of alkaline phosphatase/NBT/BCIP (blue) and diaminobenzidine/peroxidase (brown).[6] Coexpression of the two epitopes on the cells was detected by the observation of blue/brown staining.

Immunofluorescence

Immunofluorescence allows more accurate localization of an antigen within a cell than the immunoperoxidase technique and was used in this study to identify the distribution of P-selectin in the endothelial cells. The technique is similar to that of the immunoperoxidase, employing the same primary antibodies (LYP20/vWF). P-selectin expression was demonstrated with a biotinylated conjugate, followed by a streptavidin-texas red complex, giving red immunofluorescence. The indirect method was employed for vWF, with a fluorescein-conjugated secondary antibody producing a green fluorescent signal.

ICAM-1/P-Selectin Correlation

A correlation study was carried out between the expression of ICAM-1 in the arterial endothelium and the expression of P-selectin. Serial sections of artery were stained for ICAM-1, P-selectin, and vWF expression, together with MOPC21 as a negative control. Image analysis was used to obtain measurements of 160 μ lengths of intact endothelium (as identified by positive vWF expression), stained for ICAM-1 and P-selectin. One hundred measurements were made on 18 atherosclerotic plaques, nine of which were postmortem specimens and nine operative.

Results

P-Selectin Expression

Normal areas of arterial endothelium exhibited a general absence of P-selectin expression, although the adventitial blood vessels were frequently positive (Figure 1, A and B). In the endothelium overlying the atherosclerotic plaques, however, there was a distinct increase in the expression of the P-selectin adhesion molecule (Figure 1D). The degree of staining of the atherosclerotic endothelium was variable, with weak or absent staining in some areas but very strong reactions observed in others. The areas of stronger staining were usually sites of active macrophage infiltration, frequently in the shoulder regions of plaques. The levels of expression detected in the surgical and autopsy specimens were similar, with no apparent difference in reactivity or pattern of expression. Both P-selectin antibodies gave similar results. Although staining with the CLB/Thromb/6 was somewhat weaker than the LYP20 (Figure 2), the pattern of staining was identical.

The tissues used were also stained with an antibody to the platelet-specific marker, glycoprotein 1b. Platelets in occasional thrombi adherent to the vessel wall stained positively (Figure 3C) but were clearly distinguishable from the endothelium itself, which was not stained (Figure 3, A and B).

The specificity of the reaction to endothelial cells was further confirmed by double staining for

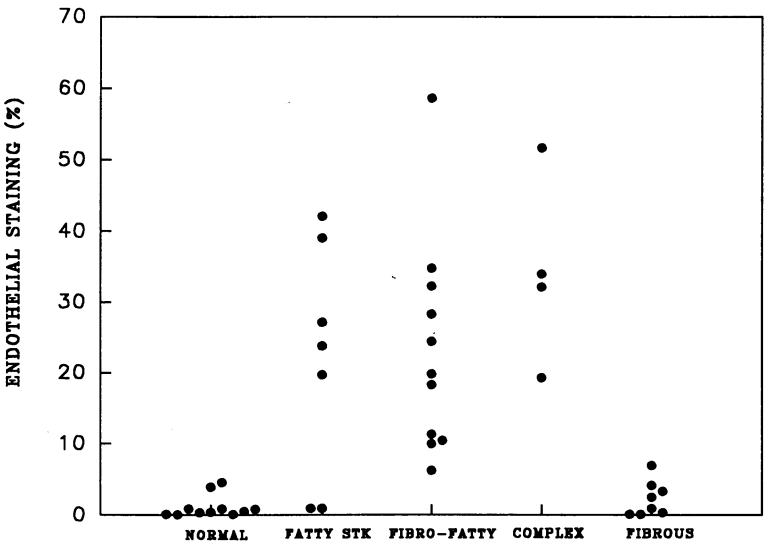

Figure 2. *Quantitative color image analysis of the endothelial layer of normal and atherosclerotic arteries stained with the P-selectin antibody LYP20, expressed as the mean percentage stained of the area analyzed.*

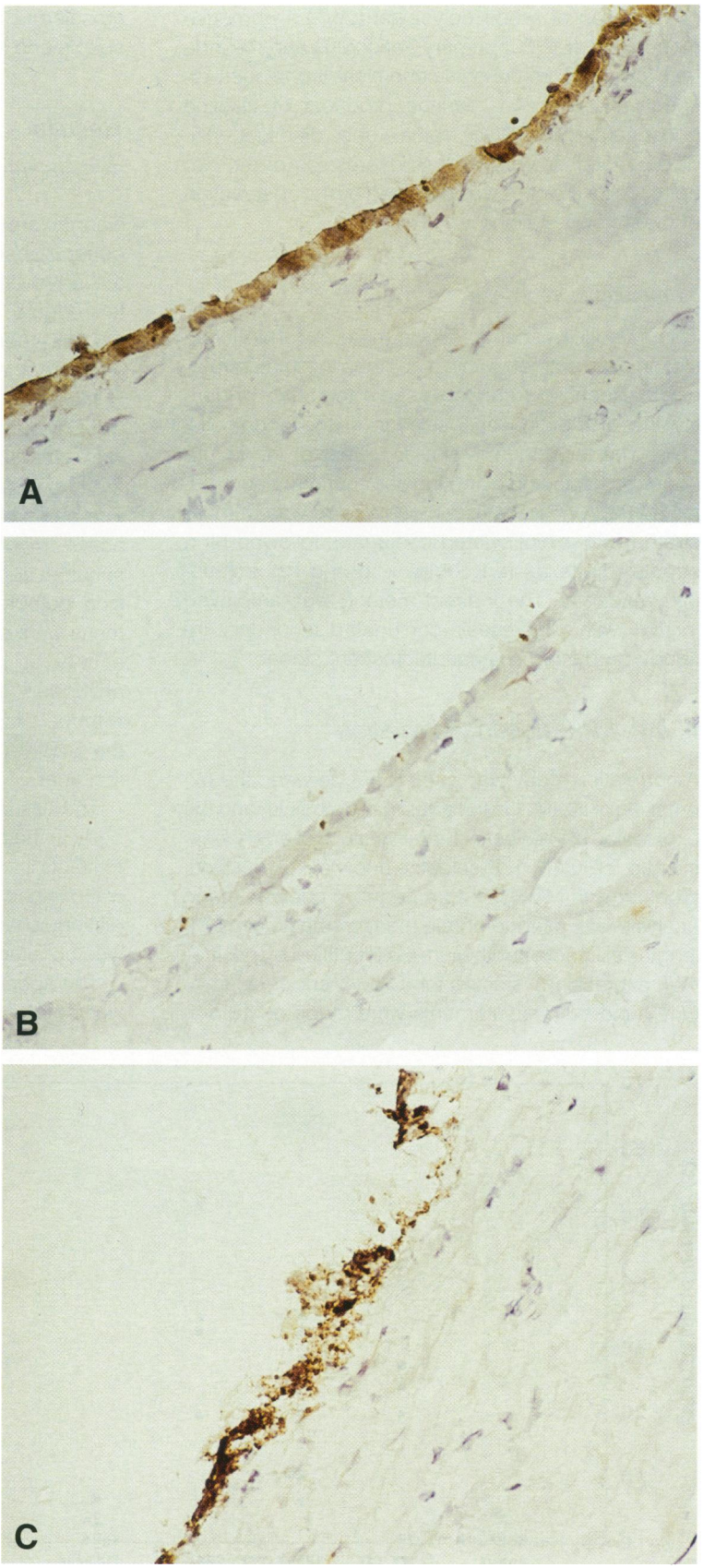

Figure 3. *Serial sections of a fibro-fatty plaque stained for P-selectin* (**A**) *and GP1b* (**B**), *showing the absence of platelets at the site of P-selectin positive endothelium. ABC immunoperoxidase, original magnification 400×.* **C** *shows GP1b-positive platelets in a mural thrombus.*

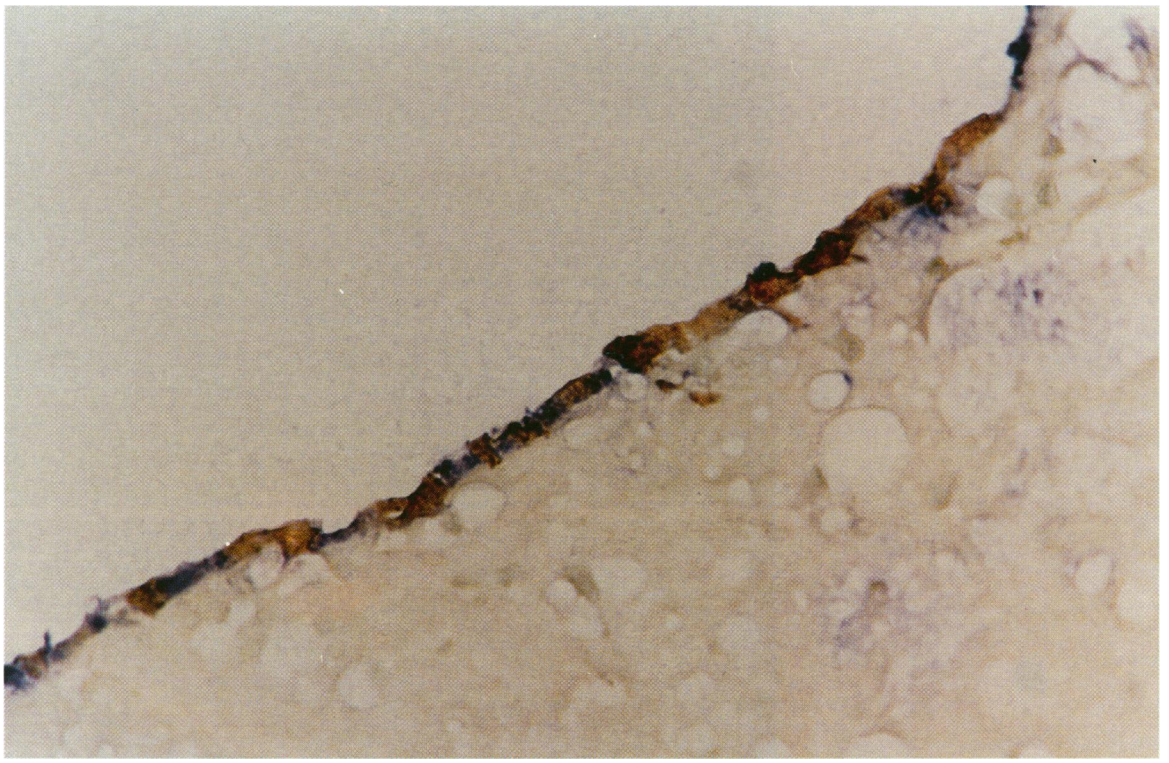

Figure 4. *Area of endothelium overlying a fibro-fatty plaque double stained to show both vWF (blue) and P-selectin (brown) expression, by alkaline phosphatase/peroxidase immunohistochemistry. Original magnification 630×.*

P-selectin and vWF, which clearly defined the endothelial layer. Individual endothelial cells were seen by double immuno-enzyme staining to be expressing both P-selectin and the endothelial-specific marker (Figure 4). Double immunofluorescence illustrates that the P-selectin detected is indeed often seen expressed on the luminal surface of the endothelial cells as well as being seen in the cytoplasm of these cells (Figure 5).

Image Analysis

Quantitative analysis of the degree of staining of the endothelium overlying the normal and atherosclerotic areas showed a significant increase with both antibodies in the expression of P-selectin in the endothelium overlying atherosclerotic plaques of fatty streaks, fibro-fatty and complex types (Mann–Whitney U test, Table 2). With the control antibody, MOPC21, minimal staining was seen, with no difference between normal and atherosclerotic areas.

ICAM-1 and P-Selectin Correlation

A highly significant correlation was found ($r = 0.84$) between the expression of ICAM-1 and P-selectin in the endothelium overlying the atherosclerotic plaques, $P < 0.001$ (Figure 1, C and D; Figure 6). Areas of plaque that express weak levels of ICAM-1 were also weakly expressive or negative for P-selectin. These areas usually had little macrophage infiltration.

Discussion

The induction of P-selectin in association with atherosclerosis is dissimilar to the mechanism of P-selectin activation previously identified in small vessels in inflammation. In the microcirculation, P-selectin is normally present within the endothelial cells, and on activation of these cells, this molecule is rapidly translocated from the Weibel–Palade bodies in the cytoplasm to the luminal surface. In this way, a functional adhesion molecule becomes activated within minutes. P-selectin is constitutively expressed in small veins and venules, but only occasional patchy distribution has been seen in small arteries and arterioles,[12] and we have also shown the absence of P-selectin expression in normal arterial endothelium. It is known, however, that P-selectin expression can be induced by activation with tumor necrosis factor-α and lipopolysaccharide

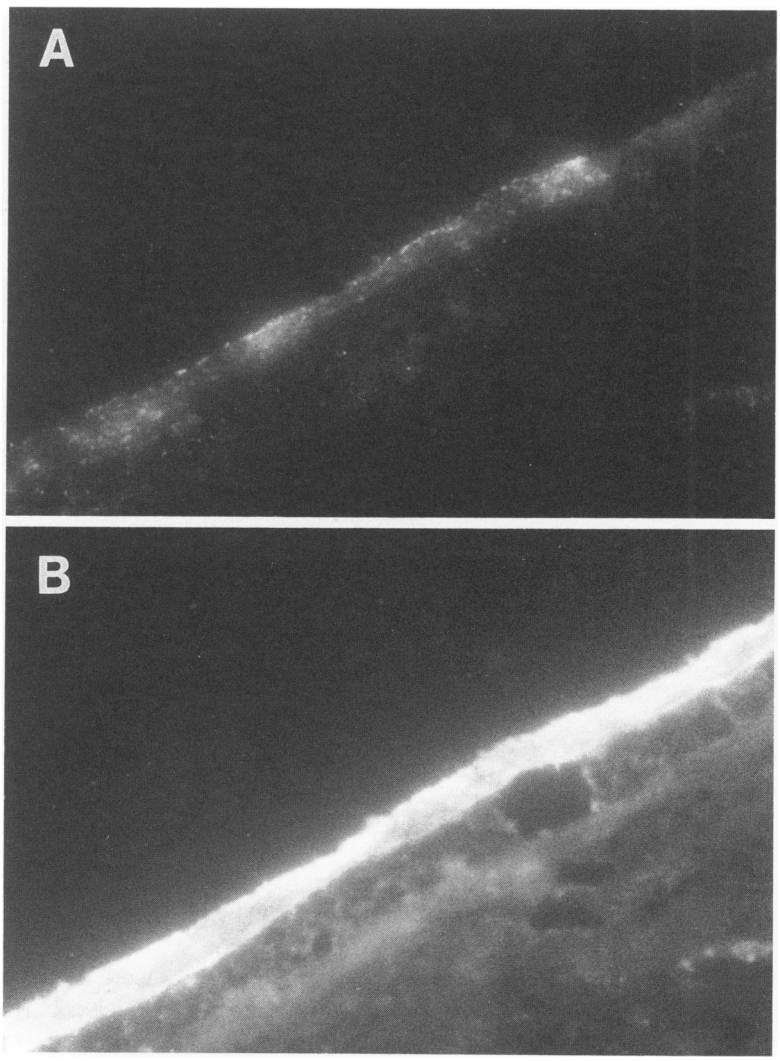

Figure 5. *Area of endothelium overlying a fibro-fatty plaque stained by double immunofluorescence to demonstrate the difference in cellular distribution of P-selectin* (**A**) *and vWF* (**B**). *P-selectin, in addition to being present in the cytoplasm, is also clearly seen expressed on the luminal surfaces of these cells. vWF is seen distributed throughout the full depth of the endothelial layer. Original magnification 630×.*

Table 2.

Lesion type	CBL-Thromb/6	LYP20	MOPC21
Normal	0.3 ± 0.1	1.1 ± 0.5	0.1 ± 0.1
Fatty streak	4.6 ± 1.8*	21.9 ± 6.2*	0.5 ± 0.3
Fibro-fatty	11.1 ± 3.2†	23.1 ± 4.6†	1.7 ± 0.5
Complex	12.0 ± 2.9*	34.9 ± 6.7*	0.6 ± 0.3
Fibrous	1.5 ± 0.5*	2.3 ± 0.9	0.2 ± 0.1
All atherosclerotic	9.2 ± 1.8†	24.8 ± 3.3†	1.0 ± 0.3

Mean percentage (± SE) of endothelial layer stained with P-selectin antibodies and the control IgG_1, MOPC21. Statistical significance (Mann–Whitney U-test) when compared to normal control artery :-

* $P < 0.01$.

† $P < 0.001$.

in cultured endothelial cells, and the majority of the newly synthesized P-selectin protein is transported to the cell surface.[31,32] Similarly, the induction of P-selectin and ICAM-1 on endothelial cells in atherosclerosis is therefore likely to imply a state of activation.

Purified P-selectin binds rapidly and reversibly to monocytes and neutrophils[26] and to some lymphocyte subsets,[12,33] as was seen initially by P-selectin mediating the adhesion of stimulated platelets to neutrophils and monocytes.[34] These properties may allow it to mediate rapid, regulated, targeting of neu-

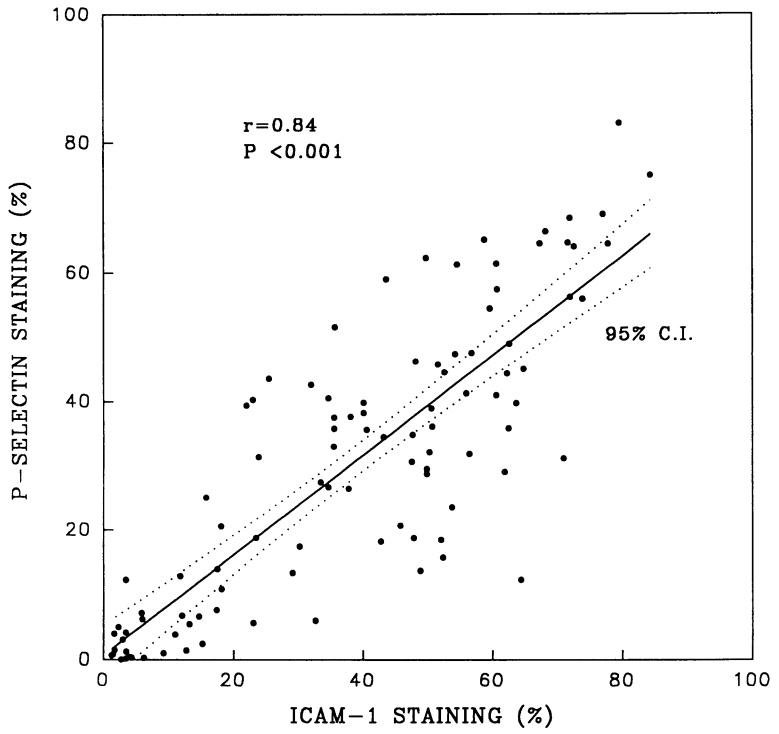

Figure 6. *Correlation between the expression of P-selectin (LYP20) and ICAM-1 (15.2) in the endothelium overlying atherosclerotic plaques. Results expressed as the mean percentage stained of the area analyzed. Dotted lines indicate 95% confidence intervals for the regression.*

trophils or monocytes to sites of inflammation. In this study, immunofluorescent staining of atherosclerotic plaques has demonstrated that P-selectin can be found expressed on the luminal surface of the endothelial cells, thus making it functionally available for leukocyte recruitment. As P-selectin expression is short-lived, this adhesion molecule is seen largely held within Weibel–Palade bodies, and the observation of its superficial expression even in advanced lesions is indicative of a continuous state of stimulation. This may be produced by the macrophage cytokines interleukin-1 and tumor necrosis factor, which have been demonstrated in the plaques.[35]

This study suggests that the accumulation of monocytes in atherosclerosis may depend in part on the synergistic action of P-selectin and ICAM-1 on the arterial endothelium, possibly in concord with other factors such as chemoattractants. P-selectin induction may be of particular significance to monocyte adhesion as it can act as a ligand for L-selectin,[36] and monocyte adhesion to activated endothelium *in vitro* has been shown to have an important component dependent on L-selectin.[37] These selectins can mediate the initial rolling contact between leukocytes and the endothelium,[22,38] which is then followed by strong adhesion and flattening dependent on ICAM-1 expressed by the endothelium. The finding that P-selectin and ICAM-1 co-distribute is therefore of particular significance. A functional role is suggested by their coincidence with areas of active infiltration of monocytes into lesions and their absence in inactive fibrous plaques.

The competence of P-selectin and ICAM-1 to induce leukocyte traffic in chronic inflammation is further supported by the findings of Miyazaki et al[39] in autoimmune thyroid disease. Small vessels were found to be positive for both P-selectin and ICAM-1, but not E-selectin or vascular cell adhesion molecule-1, and the P-selectin expression correlated with the degree of mononuclear inflammatory cell infiltration into the thyroid glands. This selective expression of P-selectin and ICAM-1 in chronic inflammation has particular relevance for atherosclerosis, as variable and mostly low levels of E-selectin and vascular cell adhesion molecule-1 have been detected in the arterial endothelium over the plaques.[7,8]

In conclusion, this work supports our previous hypothesis[6] that the focal nature of atherosclerosis may be due to lesions expanding from initiating stages by locally acting, self-perpetuating mechanisms, including monocyte infiltration and the induction of adhesion molecules on arterial endothelium. These adhesion molecules, in cooperation with other factors, such as chemoattractant cytokines and growth factors, may contribute to the recruitment of more monocytes to the lesions. In this manner, an early lesion may enhance its own development, resulting in focal plaques.

Acknowledgments

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